DESCRIPTION

GENE IMPARTING REDIFFERENTIATION ABILITY OF PLANT, AND UTILIZATION OF THE SAME

Technical Field

The present invention relates to the isolation and identification of genes that confer regenerative ability to plants, as well as methods for increasing regeneration ability and methods for selecting transformed cells, where these methods utilize these genes. The present invention allows improvement of the culture characteristics of plants, and development of transformation methods with special consideration to safety.

Background Art

Under appropriate conditions, differentiated plant tissues dedifferentiate and form calli (groups of dedifferentiated cells) after undergoing cell divisions. Depending on the conditions, calli can further redifferentiate to regenerate complete plant bodies. The ability of such differentiated cells or dedifferentiated cells to regenerate individual bodies is called totipotency, and this was initially demonstrated in the 1930s to 1950s in cultivation studies of tobacco, tomatoes, and such. Tissue culture techniques are based on this totipotency, and have been widely utilized, particularly in the field of plant breeding. For example, tissue culture techniques have been used in the production of new varieties by cell fusion and ovule culture, shortening the number of years taken for breeding and fixing of hereditary character. In recent years, tissue culture techniques have become essential for molecular breeding and basic research on plants as key techniques in artificial gene transfer (transformation methods) aimed at the functional analysis of genes.

Totipotency is generally thought to be an ability possessed by all plants. In fact, depending on the plant type, variety or organ, it is known to be easy for some plants to exhibit this ability, and difficult for others. Compared to dicotyledonous plants, the tissue culture and regeneration of monocotyledonous plants including major crops such as rice, wheat, and corn is difficult, and therefore repeated trial and error is necessary for analyses involving cultivation, including transformation methods. In rice a relatively simple culturing system has been established using the ripe seeds of specific varieties, however varieties with sufficient regenerative ability are limited. In particular, palatable varieties such as Koshihikari and Sasanishiki, and the IR line varieties widely cultivated in the tropics have low regenerative abilities, and regeneration of a plant body by tissue culturing is difficult. Improving the regenerative ability of these varieties would not only be useful for selective breeding and study

of gene characteristics, but might also lead to elucidation of the mechanism of the regenerative process. In addition, the regenerative ability of other unculturable plant species and varieties might also be improved.

Furthermore, in recent years a large number of genetically modified agricultural products (GMOs) have been developed, and their planted area is increasing year by year. At the same time, many consumers are worried about their safety. The major concern in discussions on the safety of GMOs is their incorporation of antibiotic-resistance genes. Therefore, development of transformation methods that do not use antibiotic-resistance genes will ease existing consumer concern over GMOs, and at the same time may also be advantageous to researchers as simple transformation methods that do not require expensive antibiotics.

Disclosure of the Invention

Regeneration ability is governed by the interaction of a number of genes as a quantitative trait (QTL: quantitative trait locus), but to date there have been no reports of the successful isolation of regenerative ability genes from that gene locus. An objective of the present invention is to isolate and identify genes involved with the regenerative ability of plants, and to provide methods for improving plants by utilizing these genes, and transformation methods utilizing these genes as selection markers.

Prior to breeding a hybrid population for use in detecting regenerative ability QTLs, the present inventors selected varieties to be parents of the hybrid population. They selected two varieties with a clear difference in regenerative abilities: japonica rice "Koshihikari" and indica rice "Kasalath" (photograph Fig. 1). F1 individuals were produced by crossing these two cultivars, and these were then backcrossed using Koshihikari as the recurrent parent, and self-fertilized. 99 lines of a BC1F1 population were produced, and BC1F2 seeds were collected. After using 20 BC1F2 seeds of each line to culture calli in an induction medium for 30 days, the grown calli were transferred to a regeneration medium, and this was cultivated for a further 30 days. After the 30 days, callus weight and the number of shoots per seed were measured, and average values were determined using 20 seeds of each line. This was taken to be the regenerative ability (graph Fig. 1). Genotyping of each line was carried out using 262 PCR markers. When QTL analyses relating to regenerative ability were carried out based on these data, four QTLs with the effect of increasing regenerative ability were found (Fig. 2). It was successfully found that in one of these QTLs near the TGS2451 marker on the short arm of chromosome 1 (*PSR1*; *Promoter of Shoot Regeneration 1*), the Kasalath genome had a large increasing effect on the regenerative ability of Koshihikari (Fig. 2). Next, to identify the approximate locus of the *PSR1* gene, 30 individuals whose PSR1 region had been substituted with that of Kasalath were selected from the BC2F1 population, and calli were induced using ten

seeds (BC2F2 seeds) from each of these individuals. DNAs were extracted from grown calli to determine the genotype using molecular markers, and linkage analyses were carried out by investigating regenerative ability. Furthermore, to specify the locus in detail, approximately 3,800 BC3F2 seeds in which *PSR1* segregated were used to investigate genotype using molecular markers, and high resolution linkage analysis was performed. As a result, *PSR1* was found to be located in an about 50.8 kb region between molecular markers 3132 and P182 (Fig. 3). Predictions of the genes in this region suggested the presence of four genes, including a hypothetical protein. To determine which of these genes are regenerative ability genes, a Kasalath BAC library (average length 120 kb) was constructed, and a BAC clone comprising a *PSR1* region (BHAL15) was isolated by PCR screening. Suitable restriction enzyme sites in the BHAL15 clone were used to prepare Kasalath genome fragments comprising each candidate gene region, and these were introduced to Koshihikari. As a result, it was found that the regenerative ability of Koshihikari increased only when the Kasalath genome fragment (3F in Fig. 3) comprising the gene expected to encode ferredoxin nitrite reductase (*NiR*) was introduced (Fig. 4). Ferredoxin nitrite reductase is a nitrite reductase that functions using ferredoxin as the electron donor, and has the action of converting nitrite into ammonia. The nucleotide sequences of the genetic region expected to be the ferredoxin nitrite reductase gene, and approximately 2 kb upstream thereof were determined and compared for Kasalath and Koshihikari, and many mutations were found in the nucleotide sequences (Fig. 5). Furthermore, when the expression levels of the mRNA of this gene in the calli were examined by semi-quantitative RT-PCR and real-time quantitative PCR, the amount of mRNA in Kasalath was approximately 2.5 times that in Koshihikari (top and middle rows of the photographs on the left, and the graph on the right in Fig. 6). Western blot analysis using antibodies specific to the NiR protein also showed that the NiR protein is stored in larger amounts in Kasalath than in Koshihikari (bottom row of the photographs on the left in Fig. 6). Furthermore, in a comparison of NiR enzyme activity per unit protein using the naphthyl ethylenediamine method and an NiR recombinant protein expressed in *E. coli*, the Kasalath NiR showed enzyme activity approximately 1.6 times higher than that of Koshihikari (Fig. 7). The above-mentioned results showed that the difference in regenerative ability between Koshihikari and Kasalath is primarily due to differences in the level of transcriptional regulation of the *NiR* gene, and is secondly due to differences in activity per molecule of the synthesized protein.

Introducing the genomic region of the Kasalath *PSR1* gene into Koshihikari confers regeneration ability to Koshihikari, which does not regenerate. This suggests that the Kasalath *PSR1* gene can be used as a selection marker when transforming Koshihikari. More specifically, when a vector in which the Kasalath *PSR1* gene and a target gene have been inserted in parallel is introduced into Koshihikari, only those cells to which the *PSR1* gene has been introduced will

acquire regeneration ability, and therefore regenerated plant bodies should have incorporated the target gene at the same time. To prove this notion, vectors carrying the Kasalath *NiR* genome + *35S* promoter *GUS*, Kasalath *NiR* promoter :: *NiR* cDNA :: *NiR* terminator + *35S* promoter *GUS*, rice *Actin1* promoter :: *NiR* cDNA :: *NiR* terminator + *35S* promoter *GUS* in the T-DNA region of the pBI101 binary vector, and a vector that does not carry the *NiR* gene were constructed and introduced into Koshihikari. When three types of vectors comprising the *NiR* gene were introduced, many regenerated individuals were obtained in all cases, and staining due to the *GUS* gene was observed in the calli from which they were derived (Fig. 8). In addition, the *NiR* gene has the property of metabolizing nitrite, which is toxic to plants, and utilizing this characteristic also allows the *NiR* gene to be used as a marker for transformation of highly regenerative varieties. More specifically, a vector that overexpresses the *NiR* gene under the control of an actin promoter, which is a high expression promoter in rice, was introduced into a highly regenerative Kasalath variety, and this was cultured on a medium supplemented with nitrite at a concentration that would inhibit the growth of ordinary wild types. Only transformed cells grew due to the effect of the overexpressed *NiR* gene, and GUS staining was observed only in these grown cells (Fig. 9). The use of this selection method enabled production of safer recombinant plants without the use of antibiotic resistance genes derived from microorganisms (selection markers for transformed cells), which has been considered problematic in conventional genetically modified agricultural products. Furthermore, since expensive antibiotics were unnecessary, the cost of developing the transformants was reduced.

More specifically, the present invention relates to the isolation and identification of genes that increase the regenerative ability of plants, and improvement of the cultivation characteristics of plants by utilizing these genes, and methods of transformation that use these genes as a selection marker. The present invention provides [1] to [22], described below:

[1] a DNA involved in the regeneration ability of plants, wherein the DNA is any one of (a) to (d):

(a) a DNA encoding a protein comprising the amino acid sequence of SEQ ID NO: 3;

(b) a DNA comprising a coding region of the nucleotide sequence of SEQ ID NO: 1 or 2;

(c) a DNA encoding a protein comprising an amino acid sequence with one or more amino acid substitutions, deletions, additions, and/or insertions in the amino acid sequence of SEQ ID NO: 3; and

(d) a DNA that hybridizes under stringent conditions with a DNA comprising the nucleotide sequence of SEQ ID NO: 1 or 2;

[2] a DNA encoding a partial peptide of a protein comprising the amino acid sequence of SEQ ID NO: 3;

[3] a DNA comprising a promoter region of the nucleotide sequence of SEQ ID: 1 or 2;

[4] a vector comprising the DNA of [1] or [2];

[5] a vector comprising the DNA of [3];

[6] a host cell carrying the vector of [4];

[7] a plant cell carrying the vector of [4];

[8] a plant transformant comprising the plant cell of [7];

[9] a plant transformant that is a progeny or a clone of the plant transformant of [8];

[10] a propagation material of the plant transformant of [8] or [9];

[11] a method for producing a plant transformant, wherein the method comprises the steps of introducing the DNA of [1] or [2] into a plant cell, and regenerating a plant from said plant cell;

[12] a protein encoded by the DNA of [1] or [2];

[13] a method for producing the protein of [12], wherein the method comprises the steps of culturing the host cell of [6], and collecting a recombinant protein from said cell or the culture supernatant thereof;

[14] an antibody that binds to the protein of [12];

[15] a polynucleotide comprising at least 15 continuous nucleotides that are complementary to the nucleotide sequence of SEQ ID NO: 1 or 2, or a sequence complementary thereto;

[16] a method for increasing the regeneration ability of a plant, wherein the method comprises the step of expressing the DNA of [1] or [2] in a cell of a plant;

[17] an agent for altering the regeneration ability of a plant, wherein the agent comprises the DNA of [1] or [2], or the vector of [4] as an active ingredient;

[18] a method for determining the regeneration ability of a plant cell, wherein the method comprises the step of detecting the expression of the DNA of [1] or the protein of [12] in the plant cell;

[19] a method for determining the regeneration ability of a plant cell, wherein the method comprises the step of detecting the activity of the protein of [12] in the plant cell;

[20] a method for improving the regeneration ability of a plant, wherein the method comprises the step of regulating the activity of the endogenous protein of [12] in the plant;

[21] a method for selecting a transformed plant cell, wherein the method comprises the steps of:

(a) introducing a plant cell with a vector comprising the DNA of [1] or [2] as a selection marker; and

(b) culturing the plant cell and selecting plant cells that have acquired regeneration ability; and

[22] a method for altering the regeneration ability of a plant, wherein the method comprises the step of substituting the endogenous DNA of [1] or [2] in a plant by crossing.

The present invention provides DNAs that encode rice-derived NiR protein. The nucleotide sequence of the genomic DNA of "Kasalath" is shown in SEQ ID NO: 1, the nucleotide sequence of the cDNA of "Kasalath" is shown in SEQ ID NO: 2, and the amino acid sequence of the protein encoded by the DNA is shown in SEQ ID NO: 3. The nucleotide sequence of the genomic DNA of "Koshihikari" is shown in SEQ ID NO: 4, the nucleotide sequence of the cDNA of "Koshihikari" is shown in SEQ ID NO: 5, and the amino acid sequence of the protein encoded by the DNA is shown in SEQ ID NO: 6.

The present invention showed that the regenerative ability of plants can be increased by regulating the expression or activity of the *PSR1* gene in plants. This enables culturing of unculturable varieties, such as Koshihikari, and enables production of stable and highly regenerative varieties.

The phrase "increase in regenerative ability" in the present invention means only that the ability of plants to regenerate under culturing conditions is increased, and the form of the regenerated individual is unchanged. This increase in regenerative ability allows the desired variety to be subjected to various cultivation experiments, and as a result, allows the efficient development of new varieties and functional analyses of genes.

In the present invention, the phrase "*PSR1* gene of plants" refers to the *NiR* gene encoding ferredoxin nitrite reductase of plants. "*PSR1* gene of plants" comprises the rice *PSR1* gene (Fig. 5), and *PSR1* genes derived from other plants. DNAs encoding the PSR1 protein of the present invention include genomic DNAs, cDNAs, and chemically synthesized DNAs. Genomic DNAs and cDNAs can be prepared according to conventional methods known to those skilled in the art. More specifically, genomic DNAs can be prepared, for example, as follows: (1) extract genomic DNAs from rice varieties with the *PSR1* gene (*e.g.* Koshihikari); (2) construct a genomic library (utilizing a vector such as a plasmid, phage, cosmid, BAC, and PAC); (3) develop the library; and (4) conduct colony hybridization or plaque hybridization using a probe prepared based on a DNA encoding a protein of the present invention (*e.g.* SEQ ID NO: 1 or 2). Alternatively, a genomic DNA can be prepared by PCR, using primers specific to a DNA encoding a protein of the present invention (*e.g.* SEQ ID NO: 1 or 2). On the other hand, cDNAs can be prepared, for example, as follows: (1) synthesize cDNAs based on mRNAs extracted from rice varieties with the *PSR1* gene (*e.g.* Koshihikari); (2) prepare a cDNA library by inserting the synthesized cDNAs into vectors, such as λZAP; (3) develop the cDNA library; and (4) conduct colony hybridization or plaque hybridization as described above. Alternatively, cDNAs can be also prepared by PCR.

The present invention includes DNAs encoding proteins (Kasalath) functionally equivalent to the PSR1 protein of SEQ ID NO: 3. Herein, the term "functionally equivalent to the PSR1 protein" indicates that modification of expression or activity of the object protein

results in an increase in regeneration ability.

Examples of such DNAs include those encoding mutants, derivatives, alleles, variants, and homologues comprising the amino acid sequence of SEQ ID NO: 3 wherein one or more amino acids are substituted, deleted, added and/or inserted.

Examples of methods known to those skilled in the art for preparing a DNA encoding a protein comprising altered amino acids include site-directed mutagenesis (Kramer, W. and Fritz, H. -J., (1987) "Oligonucleotide-directed construction of mutagenesis via gapped duplex DNA." Methods in Enzymology, 154: 350-367). The amino acid sequence of a protein may also be mutated in nature due to the mutation of a nucleotide sequence. DNAs encoding proteins having the amino acid sequence of a natural PSR1 protein wherein one or more amino acids are substituted, deleted, and/or added are also included in the DNAs of the present invention, so long as they encode a protein functionally equivalent to the natural PSR1 protein (SEQ ID NO: 3). Additionally, nucleotide sequence mutants that do not give rise to changes in the amino acid sequence of the protein (degeneracy mutants) are also included in the DNAs of the present invention.

DNAs encoding proteins functionally equivalent to the PSR1 protein described in SEQ ID NO: 3 can be produced, for example, by methods well known to those skilled in the art, including methods using hybridization techniques (Southern, E.M., Journal of Molecular Biology, Vol. 98, 503, 1975.); and polymerase chain reaction (PCR) techniques (Saiki, R. K. *et al.* Science, vol.230, 1350-1354, 1985; Saiki, R. K. *et al.* Science, vol.239, 487-491, 1988). That is, it is routine for a person skilled in the art to isolate DNAs with high homology to the PSR1 gene from rice and other plants by using the nucleotide sequence of the PSR1 gene (SEQ ID NO: 2) or parts thereof as a probe, and oligonucleotides hybridizing specifically to the nucleotide sequence of the PSR1 gene (SEQ ID NO: 2) as a primer. Such DNAs encoding proteins functionally equivalent to the PSR1 protein, obtainable by hybridization techniques or PCR techniques, are included in the DNAs of this invention.

Hybridization reactions to isolate such DNAs are preferably conducted under stringent conditions. Stringent hybridization conditions of the present invention include conditions such as 6 M urea, 0.4% SDS, and 0.5x SSC; and those conditions which yield similar stringencies. DNAs with higher homology are expected when hybridization is performed under conditions with higher stringency, for example, 6 M urea, 0.4% SDS, and 0.1x SSC. Those DNAs isolated under such conditions are expected to encode a protein having a high level of amino acid homology with a PSR1 protein (SEQ ID NO: 3 or 6). Herein, high homology means identity of at least 50% or more through the entire amino acid sequence, more preferably 70% or more, and much more preferably 90% or more (*e.g.* 95%, 96%, 97%, 98%, 99% or more). The degree of homology of one amino acid sequence or nucleotide sequence to another can be determined by

following the BLAST algorithm by Karlin and Altschul (Proc. Natl. Acad. Sci. USA 87:2264-2268, 1990; Proc. Natl. Acad. Sci. USA, 90: 5873, 1993). Programs such as BLASTN and BLASTX were developed based on the BLAST algorithm (Altschul SF, *et al.* J. Mol. Biol.215: 403, 1990). To analyze a nucleotide sequences according to BLASTN, the parameters are set as score=100 and word length=12, for example. On the other hand, parameters used for the analysis of amino acid sequences by BLASTX include, for example, score=50 and word length=3. The default parameters for each program are used when using BLAST and Gapped BLAST program. Specific techniques for such analyses are known in the art.

Whether a particular DNA encodes a protein involved in the regeneration ability of a plant can be evaluated as follows. The most conventional methods involve deleting the function of a DNA, then cultivating, and investigating the ability to regenerate. More specifically, the methods involve cultivating under conditions where the function of a DNA is maintained, and under conditions where the function of a DNA is deleted, and comparing the resulting regeneration abilities. If the regeneration abilities do not change or are nearly the same, the DNA is not involved in regeneration ability. When the DNA is involved in regeneration ability, the regeneration ratio is further increased, and this difference is considered to be the degree of regeneration ability.

The DNAs of the present invention can be used, for example, to prepare recombinant proteins, and to produce plant transformants having altered regeneration abilities. A recombinant protein is usually prepared by inserting a DNA encoding a protein of the present invention into an appropriate expression vector, introducing the vector into an appropriate cell, culturing the transformed cells, allowing the cells to express the recombinant protein, and purifying the expressed protein. A recombinant protein can be expressed as a fusion protein with other proteins so as to be easily purified, for example, as a fusion protein with maltose binding protein in *Escherichia coli* (New England Biolabs, USA, vector pMAL series), as a fusion protein with glutathione-S-transferase (GST) (Amersham Pharmacia Biotech, vector pGEX series), or tagged with histidine (Novagen, pET series). The host cell is not limited so long as the cell is suitable for expressing the recombinant protein. It is possible to utilize yeasts or various animal, plant, or insect cells as well as the above described *E. coli*. A vector can be introduced into a host cell by a variety of methods known to one skilled in the art. For example, a transformation method using calcium ions can be used to introduce a vector into *E. coli* (Mandel, M. and Higa, A. (1970) Journal of Molecular Biology, 53, 158-162, Hanahan, D. (1983) Journal of Molecular Biology, 166, 557-580). A recombinant protein expressed in host cells can be purified and recovered from host cells or the culture supernatant thereof by known methods. When a recombinant protein is expressed as a fusion protein with maltose binding

protein or other partners, the recombinant protein can be easily purified by affinity chromatography. A protein of the present invention can be prepared from transformed plants which have been generated by introducing a DNA of this invention into plants as described below. Thus, as described below, the transformed plants of the present invention include not only plants with a DNA of this invention introduced to alter their regeneration ability, but also plants with a DNA of this invention introduced to prepare a protein of this invention.

The resulting proteins can be used to prepare antibodies that bind to the proteins. For example, a polyclonal antibody can be prepared by immunizing immune animals, such as rabbits, with a purified protein of the present invention or a portion thereof, collecting blood after a certain period, and removing clots. A monoclonal antibody can be prepared by fusing myeloma cells with the antibody-forming cells of animals immunized with the above protein or its portion, isolating monoclonal cells that express a desired antibody (hybridomas), and recovering the antibodies from the cell. The obtained antibodies can be utilized to purify or detect a protein of the present invention. Accordingly, the present invention includes antibodies that bind to proteins of the invention. The use of these antibodies enables one to distinguish the expression site of proteins involved in the regeneration ability of a plant body, or to determine whether a plant species expresses a protein involved in regeneration ability.

When producing a transformed plant in which regeneration ability has been increased by utilizing a DNA of this invention, a DNA encoding a protein of this invention is inserted into an appropriate vector, which is then introduced into a plant cell. The transformed plant cells obtained by these steps are then regenerated. Plant cells to which the vector is introduced are preferably plant cells with low expression of the DNA of the present invention. Herein, the term "plant cells" includes plant cells of various forms, such as suspension culture cells, protoplasts, leaf sections, and calli.

Vectors used for plant cell transformation are not particularly limited as long as they can express the inserted genes in the cells. Examples include the "pBI121", "pBI221", and "pBI101" plasmids (all from Clontech).

The vectors of this invention may comprise a promoter for constitutively or inductively expressing the proteins of this invention. Examples of promoters for constitutive expression include the 35S promoter of cauliflower mosaic virus (Odell *et al.* 1985 Nature 313:810), actin promoter of rice (Zhang *et al.* 1991 Plant Cell 3:1155), and ubiquitin promoter of corn (Cornejo *et al.* 1993 Plant Mol. Biol. 23:567).

Examples of promoters for inductive expression include promoters known to initiate expression due to extrinsic factors, such as infection and invasion of filamentous fungi, bacteria, and viruses, low temperature, high temperature, dryness, ultraviolet irradiation, and spraying of particular compounds. Examples of such promoters include the chitinase gene promoter of rice

(Xu *et al.* 1996 Plant Mol. Biol. 30:387) and the tobacco PR protein gene promoter (Ohshima *et al.* 1990 Plant Cell 2:95), which are induced by infection and invasion of filamentous fungi, bacteria, and viruses, the "lip19" gene promoter of rice, which induced by low temperature (Aguan *et al.* 1993 Mol. Gen Genet. 240:1), the "hsp 80" gene and "hsp 72" gene promoters of rice, which are induced by high temperature (Van Breusegem *et al.* 1994 Planta 193:57), the "rab 16" gene promoter of *Arabidopsis thaliana*, which is induced by dryness (Nundy *et al.*, 1990 Proc. Natl. Acad. Sci. USA 87:1406), chalcone synthase gene promoter of parsley, which is induced by ultraviolet irradiation (Schulze-Lefert *et al.* 1989 EMBO J. 8:651), and the alcohol dehydrogenase gene promoter of corn, which is induced by anaerobic conditions (Walker *et al.*, 1987 Proc. Natl. Acad. Sci. USA 84:6624). In addition, the chitinase gene promoter of rice and PR protein gene promoter of tobacco can also be induced by specific compounds such as salicylic acid, and the "rab 16" can also be induced by spraying abscisic acid, a phytohormone.

In addition, the vectors may comprise a promoter of a DNA encoding a protein of the invention. A promoter region of a DNA encoding a protein of the invention can be obtained by, for example, screening a genomic library using a DNA comprising the nucleotide sequence of SEQ ID NO: 1 or 2, or a portion thereof, as a probe.

Furthermore, the present invention provides transformed cells to which a vector of this invention has been introduced. In addition to the above-mentioned cells used for producing recombinant proteins, the cells to which a vector of this invention is introduced include plant cells for preparing transformed plants. There are no particular limitations as to the type of plant cells, and examples are cells of *Arabidopsis thaliana*, rice, corn, potato, and tobacco. In addition to cultured cells, the plant cells of this invention include cells within plants, and also protoplasts, shoot primordia, multiple shoots, and hairy roots. Vectors can be introduced into plant cells by known methods, such as polyethylene glycol methods, electroporation, *Agrobacterium* mediated transfer, and particle bombardment. Plants can be regenerated from transformed plant cells by known methods, depending on the type of plant cell (Toki *et al.*, (1995) Plant Physiol. 100:1503-1507). For example, transformation and regeneration methods for rice plants include: (1) introducing genes into protoplasts using polyethylene glycol, and regenerating the plant body (suitable for indica rice varieties) (Datta, S.K. (1995) in "Gene Transfer To Plants", Potrykus I and Spangenberg Eds., pp66-74); (2) introducing genes into protoplasts using electric pulse, and regenerating the plant body (suitable for japonica rice varieties)(Toki *et al.* (1992) Plant Physiol. 100, 1503-1507); (3) introducing genes directly into cells by particle bombardment, and regenerating the plant body (Christou *et al.* (1991) Bio/Technology, 9: 957-962); and (4) introducing genes using *Agrobacterium*, and regenerating the plant body (Hiei *et al.* (1994) Plant J. 6: 271-282). These methods are already established in the art and are widely used in the technical field of the present invention. Such methods can

be suitably used for the present invention.

Having obtained a transformed plant containing a DNA of the present invention in its genome, it is possible to obtain a progeny of the plant by sexual or asexual reproduction. It is also possible to obtain reproductive material (such as seeds, fruits, spikes, tubers, tuberous roots, stubs, calli, and protoplasts) from the plant or a progeny or clone thereof, to mass-produce the plant based on such material. Thus, the present invention includes plant cells to which the DNA of the present invention has been introduced, plants containing these cells, progenies and clones of these plants, as well as reproductive material of the plants and their progenies and clones.

Plants produced in this manner whose regeneration ability has been modified show changes in their regeneration ability and yield as compared to wild-type plants. For example, plants in which a DNA encoding PSR1 protein has been introduced under the control of rice actin promoter are expected to show an increase in their regeneration abilities. Use of the methods of this invention can increase the regeneration ability of rice, which is a useful agricultural crop. The present invention is further beneficial in the development of highly regenerative rice varieties.

Furthermore, the present invention provides polynucleotides comprising at least 15 continuous nucleotides, which are complementary to the nucleotide sequence of SEQ ID NO: 1 or 2, or their complementary sequences. Herein, the phrase "complementary sequence" refers to a sequence of one strand with respect to the sequence of the other strand of a double-stranded DNA comprising A:T and G:C base pairs. The term "complementary" is not limited to cases in which a sequence is completely complementary to a region of at least 15 continuous nucleotides, and includes cases in which nucleotide sequence identity is at least 70%, preferably at least 80%, more preferably 90%, and even more preferably 95% or more (for example, 96% or more, 97% or more, 98% or more, or 99% or more). Such DNAs are useful as probes for detecting or isolating the DNAs of this invention, and as primers for amplifying the DNAs.

The present invention also provides methods of genetic diagnosis for determining the presence of regeneration ability in plants. In the present invention, "determining the presence of regeneration ability in plants" is not only effective for determining the presence of regeneration ability in varieties that have been cultivated so far, but also includes determining the presence of regeneration ability in new varieties produced by crossing and genetic engineering techniques. These methods are particularly effective for determining the presence of regeneration ability in japonica rice varieties.

The methods of the present invention for evaluating the presence of regeneration ability in plants comprise detection of plant expression levels of DNAs encoding the PSR1 protein, and of the PSR1 protein. For example, if the level of expression of a DNA encoding PSR1, or of

the PSR1 protein, is higher than in Koshihikari, the examined plant is determined to be a variety possessing regeneration ability.

The present invention provides methods for utilizing the *PSR1* gene as a selection marker in the transformation of plants. Examples of previously used selection marker genes of transformed plant cells include the hygromycin phosphotransferase gene that gives resistance to the antibiotic hygromycin, neomycin phosphotransferase that gives resistance to kanamycin or gentamycin, acetyl transferase gene that gives resistance to the herbicide phosphinothricin, and bialaphos resistance gene that gives resistance to bialaphos. When using these genes, transformed plant cell cultures are obtained by culturing in a known selection medium containing a selection agent that is suited to the type of selection marker gene. When using the *PSR1* gene as a selection marker, instead of these drug-resistance genes, if the plant cells to be transformed do not have regeneration ability, as in Koshihikari, transformants can be selected using the acquired regeneration ability as a marker trait, without the use of special agents and such for selection. That is, since non-transformants cannot regenerate, individuals that regenerated due to the effect of the *PSR1* gene are assumed to be transformants. Furthermore, when utilizing the PSR1 gene as a selection marker for plant cells with regeneration ability, the transformed cells can be selected by adding a certain concentration of nitrite, which would inhibit the growth of non-transformants, to the selection media. The above-mentioned conventional drug resistance genes used to select transformants are derived from microorganisms; therefore, genetically modified agricultural products (GMOs) in which such genes remain have raised concerns regarding adverse effects on the ecosystem and on the human body. However, the methods for selecting transformants that use the *PSR1* gene of this invention have advantages in that such concerns can be relieved and inexpensive genetically modified crops can be developed.

All prior art documents cited herein are incorporated by reference.

Brief Description of the Drawings

Fig. 1 is a graph and a set of photographs indicating the phenotypes of Koshihikari and Kasalath. The photograph on the left shows Koshihikari, and the photograph on the right shows Kasalath. The graph indicates the regeneration ability of Koshihikari and Kasalath as the number of regenerated individuals per gram of calli.

Fig. 2 shows the positions of regeneration ability QTLs on the chromosome.

Fig. 3 shows a highly accurate linkage map of the regeneration ability QTLs.

Fig. 4 is a set of photographs indicating the results of complementation tests. The left photograph shows the result when the vector alone is inserted into Koshihikari, while the right photograph shows the regeneration that occurs when the 3F fragment of Kasalath is inserted into Koshihikari.

Fig. 5 shows the mutation sites of the Kasalath *NiR* genome compared to the Koshihikari *NiR* genome sequence. The Arabic numerals in the schematic diagram indicate the number of inserted or deleted nucleotides. Black squares indicate coding regions. Vertical lines indicate substitution sites. The framed part shows comparison of the *NiR* gene sequences in Koshihikari (top) and Kasalath (bottom). The parts enclosed in boxes indicate the amino acids that were different between Koshihikari and Kasalath. The region indicated in bold italics indicates the chloroplast transit peptide domain, the region indicated by the dotted underline indicates the ferredoxin binding region, and the underlined portion indicates the 4Fe-4S cluster.

Fig. 6 is a set of photographs and a graph comparing the expression levels of the *NiR* genes and NiR proteins in the calli of Koshihikari and Kasalath. In the left photograph the top row shows the *NiR* gene as detected by semi-quantitative RT-PCR, the middle row shows the rice ubiquitin 1 gene (*Rubq1*), used as an expression control and detected by semi-quantitative RT-PCR, and the lower row shows the NiR protein as detected by Western blot hybridization using the NiR protein antibody. The graph on the right shows the results of measuring the expression level of the *NiR* genes by realtime quantitative RT-PCR using the expression level of the *Rubq1* gene as an internal standard. The RT-PCR primer sites are shown in Fig. 5.

Fig. 7 is a graph comparing the enzyme activities of the Koshihikari and Kasalath NiR recombinant proteins.

Fig. 8 is a diagram and a set of photographs showing the results of an experiment for confirming the effectiveness of the *NiR* gene as a selection marker. The schematic diagram shows the T-DNA region of the binary vector used for transformation. The photographs show the state of regeneration when each vector is introduced into Koshihikari. The table shows the proportion of GUS-stained individuals among the regenerated individuals.

Fig. 9 is a photograph showing the result of selecting calli when a vector that overexpresses the *NiR* gene by the actin promoter is introduced into Kasalath. The top photograph shows the result of callus selection. Since nitrite was added to the medium, transformant "a" grew due to the effect of the overexpressed *NiR* gene, whereas callus growth of non-transformant "b" was inhibited. The bottom photographs show the GUS staining results for calli "a" and "b".

Best Mode for Carrying Out the Invention

Herein below, the present invention will be specifically described using examples, however, it is not to be construed as being limited thereto.

[Example 1] Selection of test material and production of near-isogenic lines

Prior to breeding a hybrid population for use in QTL analysis, varieties were selected to

be the hybrid population parents. First, the average regeneration ability of several varieties of japonica rice and several varieties of indica rice were studied, and two varieties with a clear difference in regeneration abilities were selected: japonica rice "Koshihikari" and indica rice "Kasalath" (photograph Fig. 1). F1 individuals were produced by crossing japonica variety "Koshihikari" and indica variety "Kasalath". These individuals were then backcrossed using Koshihikari as the recurrent parent, and self-fertilized. After producing the BC1F1 population, BC1F2 seeds were collected. 20 BC1F2 seeds from each line were used to culture the calli in an induction medium for 30 days, then grown calli were transferred to a regeneration medium. 30 days after transfer, the callus weight and number of shoots per seed were measured, and average values were determined using the 20 seeds of each line. This was taken to be the regeneration ability (graph Fig. 1). Genotypes of each line were determined using 262 PCR markers.

When QTL analyses relating to regeneration ability were carried out based on these data, four QTLs having the effect of increasing regeneration ability were found (Fig. 2). It was successfully found that in one of these QTLs near the TGS2451 marker on the short arm of chromosome 1 (*PSR1*; *Promoter of Shoot Regeneration 1*), the Kasalath genome had a large increasing effect on the regeneration ability of Koshihikari. *PSR1* near-isogenic line (Nil-PSR1: a line in which a substitution has been made on the Koshihikari chromosome using a region near the Kasalath chromosome 1 TGS2451 marker) was produced using repeated backcrossing and MAS. The regeneration ability of Nil-PSR1 and Koshihikari (control) was investigated, and the presence of QTL (PSR1) was confirmed. In the line in which the region near TGS2451 on the short arm of chromosome 1 had been substituted with that of Kasalath, regeneration ability increased an average of 14.7 times.

[Example 2] High resolution linkage analysis using a segregating population of PSR1

30 individuals whose *PSR1* region had been substituted with that of Kasalath were selected from the BC2F1 population. Ten of each seed (BC2F2 seeds) were used, and DNA was extracted from the calli. The genotype was elucidated using molecular markers, and linkage analyses were carried out by investigating regeneration ability. Furthermore, to specify the locus in detail, approximately 3,800 BC3F2 seeds in which *PSR1* segregated were used to investigate genotype using molecular markers, and high resolution linkage analysis was performed. As a result, *PSR1* was found to be located in an about 50.8 kb region between molecular markers 3132 and P182 (Fig. 3). Predictions of genes in this region suggested the presence of four genes, including a hypothetical protein. To determine which of these genes are regeneration ability genes, a Kasalath BAC library (average length 120 kb) was constructed, and a BAC clone comprising a *PSR1* region (BHAL15) was isolated by PCR screening. Suitable

restriction enzyme sites in the BHAL15 clone were used to prepare Kasalath genome fragments comprising each candidate gene region, and these were introduced to Koshihikari. As a result, it was found that the regeneration ability of Koshihikari increased only when the Kasalath genome fragment (3F in Fig. 3) comprising the gene expected to encode ferredoxin nitrite reductase (*NiR*) was introduced (Fig. 4). The nucleotide sequences of the genetic region predicted to encode ferredoxin nitrite reductase and the approximately 2 kb upstream thereof were determined and compared for Kasalath and Koshihikari, and many mutations were found in the nucleotide sequences (Fig. 5).

[Example 3] Improving the culturing characteristics of unculturable varieties

The *PSR1* gene region of Kasalath (either the genomic sequence or cDNA sequence may be used) was introduced into Koshihikari to confer regeneration ability to Koshihikari, yielding highly regenerative Koshihikari (Figs. 4, 8, and 9). In this case, both PSR promoter and a constitutive promoter such as actin promoter were effective as a promoter used for expressing the *PSR1* gene.

[Example 4] Expression analysis of the *PSR1* gene and PSR1 protein

When the expression levels of the *NiR* mRNA in calli were examined by semi-quantitative RT-PCR and real-time quantitative PCR, the amount of mRNA in Kasalath was approximately 2.5 times that in Koshihikari (top and middle rows of the photographs on the left, and the graph on the right in Fig. 6). Western blot analysis using antibodies specific to the NiR protein also showed that the NiR protein is stored in larger amounts in Kasalath than in Koshihikari (bottom row of the photographs on the left in Fig. 6). Furthermore, in a comparison of NiR enzyme activity per unit protein using the naphthyl ethylenediamine method and an NiR recombinant protein whose expression is induced by *E. coli*, the Kasalath NiR protein showed enzyme activity approximately 1.6 times higher than that of Koshihikari (Fig. 7). The above-mentioned results showed that the difference in regeneration ability between Koshihikari and Kasalath is primarily due to the difference in the level of transcriptional regulation of the *NiR* gene, and is secondly due to differences in activity per molecule of the synthesized protein.

[Example 5] Transformation that uses regeneration ability as the selection trait

Introduction of the Kasalath *PSR1* gene into Koshihikari can confer regeneration ability to Koshihikari, which does not regenerate. This indicates that Kasalath *PSR1* gene can be used as a selection marker when transforming Koshihikari. More specifically, when a vector in which the Kasalath *PSR1* gene and a target gene have been inserted tandemly is introduced into

Koshihikari, only those cells to which the *PSR1* gene has been introduced will acquire regeneration ability. Therefore, regenerated plant bodies should have incorporated the target gene at the same time. To prove this notion, vectors carrying the Kasalath *NiR* genome + *35S* promoter *GUS*, Kasalath *NiR* promoter:: *NiR* cDNA :: *NiR* terminator + *35S* promoter *GUS*, rice *Actin1* promoter :: *NiR* cDNA :: *NiR* terminator + *35S* promoter *GUS* in the T-DNA region of the pBI101 binary vector, and a vector that does not carry the NiR gene were constructed, and introduced into Koshihikari. As a result, when three types of vectors comprising the *NiR* gene were introduced, many regenerated individuals were obtained, and staining due to the GUS gene was observed in the calli from which they were derived (Fig. 8).

In addition, the *NiR* gene has the property of metabolizing nitrite, which is toxic to plants, and utilizing this characteristic also allows the *NiR* gene to be used as a marker for transformation of highly regenerative varieties. More specifically, a vector that overexpresses the *NiR* gene under the control of an actin promoter, which is a high expression promoter in rice, was introduced into a highly regenerative Kasalath variety, and this was cultured on a medium supplemented with nitrite at a concentration that would inhibit the growth of ordinary wild types. Only transformed cells grew due to the effect of the overexpressed *NiR* gene, and GUS staining was observed only in these grown cells (Fig. 9). The use of this selection method enabled the cost of antibiotics to be reduced compared to conventional methods in which antibiotic resistance genes derived from microorganisms are used as selection markers. Additionally, this method enabled production of more environmentally-friendly recombinant plants since the regenerated plants do not contain microorganism genes.

Industrial Applicability

Recently, studies utilizing transformation methods for the development of useful plants and for functional analyses of genes are progressing rapidly. Since transformation methods allow the use of genes beyond the confines of biological species, which is impossible in conventional breeding based on crossing and selection, novel plants may be produced. Furthermore, as genomic sequences are elucidated one after another, transformation methods are also being used for gene disruption, expression regulation analysis, and such to elucidate the function of each gene. Generally, when producing a plant transformant, a plasmid vector comprising both the gene to be introduced and a drug resistance marker gene such as an antibiotic resistance gene is introduced into plant cells by the Agrobacterium method or by electroporation, and transformed cells are selected by drug treatment. The transformed cells that are selected regenerate into plant bodies through cell growth. Thus, to utilize such transformation methods, tissue culturing techniques must be established. Tissue culturing techniques are extremely useful not only in transformation methods, but also in mutant

production using somaclonal variation, cultivar breeding using cell fusion or ovule culture, fixation of hereditary character, shortening of the number of years taken for breeding, and the like.

The major grain for which culturing techniques are most utilized is rice, but the presence of large differences in culturing characteristics between varieties is considered a problem. In particular, it is difficult to culture the major varieties in Japan, such as Koshihikari and Akitakomachi, as well as many indica varieties cultivated in the tropics, and therefore these varieties cannot be used as materials for tissue cultures. These differences in culturing characteristics between varieties are phenomena commonly observed in a number of plants and is not limited to rice, but there has been no progress in elucidating their causes.

The present inventors isolated genes involved in regeneration ability, enabling efficient selection of highly regenerative traits by using molecular markers (marker selected breeding), and enabling improvement of regeneration ability using molecular biological methods (molecular breeding). Furthermore, utilization of the *PSR1* gene as a selection marker has enabled the production of inexpensive and environmentally considerate plant transformants.

Grains such as rice, corn, wheat, and barley are major energy sources for humans, and are the most important plants for humans. These grains all belong to the family *Poaceae*, and seem to have evolved from a common ancestor. They have high genetic homology (genomic synteny) with one another. Of these grains, rice has the smallest genome, and this is why rice is used as a model plant for grains. Rice genes are present in the genomes of rice relatives such as wheat and corn, and genes isolated from rice can be easily isolated from wheat and corn. In addition, rice genes can be applied directly to grain breeding of wheat, corn, and such. Therefore, the present genes may be applied not only to rice but also to wide varieties of plants.